Symposium

The Nature and Application of Biocontrol Microbes III: *Pseudomonas* spp.

Induced Systemic Resistance by Fluorescent *Pseudomonas* spp.

Peter A. H. M. Bakker, Corné M. J. Pieterse, and L. C. van Loon

Institute of Environmental Biology, Faculty of Science, Utrecht University, P.O. Box 80084, 3508 TB Utrecht, The Netherlands.

ABSTRACT

Bakker, P. A. H. M., Pieterse, C. M. J., and van Loon, L. C. 2007. Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97:239-243.

Fluorescent *Pseudomonas* spp. have been studied for decades for their plant growth-promoting effects through effective suppression of soilborne plant diseases. The modes of action that play a role in disease suppression by these bacteria include siderophore-mediated competition for iron, antibiosis, production of lytic enzymes, and induced systemic resistance (ISR). The involvement of ISR is typically studied in systems in which the *Pseudomonas* bacteria and the pathogen are inoculated and remain spatially separated on the plant, e.g., the bacteria on the root and the pathogen on the leaf, or by use of split root systems. Since no direct interactions are possible between the two populations, suppression of disease development has to be plant-mediated. In this review, bacterial traits involved in *Pseudomonas*-mediated ISR will be discussed.

Induced resistance is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli (42). In 1991, the research groups of B. Schippers in Baarn, The Netherlands, and J. W. Kloepper in Auburn, AL, discovered independently that induced systemic resistance (ISR) is a mode of action of plant growth-promoting rhizobacteria (PGPR), especially fluorescent pseudomonads, in suppressing diseases (43,49). The *Pseudomonas* bacteria were inoculated into the rhizosphere and remained spatially separated from the pathogen that was inoculated on the aboveground plant parts, either into the stem (43) or on the leaf surface (49). By ensuring spatial separation between the *Pseudomonas* bacteria and the pathogen on the root system, for instance in a split root system, it was demonstrated that ISR is also effective against root-infecting pathogens (15,52). A threshold population density of 10^5 colony forming units per gram of root was required for effectiveness of the resistance-inducing *Pseudomonas* strain (32). When used under commercial greenhouse conditions, the ISR triggering *P. fluorescens* strain WCS374r significantly protected radish from Fusarium wilt leading to average yield increases of 40% (17).

In the last decade it has become clear that elicitation of ISR is a widespread phenomenon, not only for fluorescent pseudomonads but for a variety of nonpathogenic microorganisms and biological control agents. ISR is phenotypically similar to systemic acquired resistance (SAR) that is triggered by necrotizing pathogens in that disease caused by a challenging pathogen is reduced. Accumulation of salicylic acid (SA) in the plant is required for SAR (40). In transgenic plants that constitutively express the *nahG* gene, a salicylate hydroxylase gene from *P. putida*, SA cannot accumulate and, accordingly, these plants no longer express SAR (8). The SA-dependent SAR pathway can also be triggered by applying SA exogenously. In *Arabidopsis thaliana*, ISR by *P. fluorescens* WCS417r is independent of SA accumulation as it is still operative in NahG plants (28). From experiments with mutants of *Arabidopsis* that are non- or less responsive to ethylene or jasmonic acid (JA), it was concluded that an intact response to these plant hormones is required for expression of ISR, whereas SAR is still expressed in these mutants (29). Both ISR and SAR are effective against a wide range of pathogens. However, there are differences in the effectiveness of the signaling compounds involved against different types of pathogens. In *Arabidopsis*, SAR is effective against pathogens that in noninduced plants are resisted through SA-dependent defenses, whereas ISR is effective against pathogens that are resisted through JA/ethylene-dependent defenses (41). This suggested that the range of pathogens that are controlled might be extended when ISR and SAR are combined. Interestingly, when ISR and SAR are activated simultaneously, enhanced disease suppression occurs against pathogens against which both SA and JA/ethylene responses are effective, such as *P. syringae* pv. *tomato* (45). Thus, there appears to be room to increase the effectiveness of induced resistance. To manipulate this phenomenon effectively for practical applications, knowledge on bacterial traits involved in the triggering of ISR is essential. In this paper, we will focus on the determinants of *Pseudomonas* that are involved in triggering ISR (an overview of the determinants is presented in Table 1) and discuss future directions of research.

Flagella. Bacterial flagellins, the main protein component of flagella, can elicit defense responses in plants (9,53). For *P. putida* strain WCS358, the involvement of flagella in ISR was studied in *Arabidopsis*, bean, and tomato by applying isolated flagella and by using nonmotile mutants that lack flagella (23). In *Arabidopsis*, application of WCS358 flagella triggered ISR against *P. syringae* pv. *tomato*, whereas in bean or tomato, their application did not lead to induced resistance. Moreover, a mutant of WCS358 that lacks flagella was as effective in triggering ISR in *Arabidopsis* as the parental strain, and it was concluded that there are additional determinants in strain WCS358 that can induce resistance. These results suggest that although flagella can be involved in ISR, they are not the main trigger for this *Pseudomonas* strain.

Lipopolysaccharides. Reports that cell surface components of pathogenic bacteria can induce resistance (10,24) stimulated a study on the possible involvement of lipopolysaccharides (LPS)

Corresponding author: P. A. H. M. Bakker
E-mail address: P.A.H.M.Bakker@bio.uu.nl

DOI: 10.1094/PHYTO-97-2-0239

in ISR by *P. fluorescens* WCS417r against *Fusarium oxysporum* f. sp. *dianthi* in carnation (44). Heat-killed cells and purified LPS of WCS417r applied to the carnation roots triggered ISR to a level similar to that obtained by treatment with viable bacterial cells. Accumulation of phytoalexins after challenge inoculation of carnation treated with either viable or heat-killed cells or with LPS of WCS417 was significantly increased compared with that in control plants that were challenged with *Fusarium* spp. In potato, application of live cells or extracted LPS of *Rhizobium etli* G12 triggered ISR against *Globodera pallida*, indicating that LPS plays an important role in this system (36). In radish, LPS was also demonstrated to be of importance in ISR by *P. fluorescens* strains WCS374r and WCS417r against wilt caused by *F. oxysporum* f. sp. *raphani* (16). In this study, evidence for the involvement of the LPS was based both on applying the isolated LPS and on the use of mutants of the strains that lack the 0-antigenic side chain of the LPS. The 0-antigen minus mutants did not reduce disease incidence, whereas application of LPS did reduce Fusarium wilt of radish to a level comparable to that after treatment with the wild-type bacteria. In *A. thaliana*, LPS of *P. fluorescens* WCS417r and *P. putida* WCS358 appears to be involved in ISR against *P. syringae* pv. *tomato*, since applying isolated LPS triggered ISR. However, in this system, mutants lacking the 0-antigen were as effective as the parental strain, suggesting redundancy in ISR triggering traits in these strains (2,23,46). In bean and tomato, a mutant of WCS358 lacking the 0-antigen no longer induced resistance, and application of LPS did trigger ISR (23).

At first, the involvement of LPS in *P. fluorescens*-mediated ISR in radish appeared to be straightforward (16). However, redundancy of ISR triggering traits in *P. fluorescens* strains WCS374 and WCS417 has been reported for the suppression of Fusarium wilt in radish (14). Under conditions of high iron availability, mutants of the *P. fluorescens* strains that lack the 0-antigenic side chain of the LPS do not trigger ISR. However, upon lowering the iron availability in the plant root system, the wild-type strains were more effective and the mutants became as effective as the wild-type strains. These data suggested that additional iron-regulated metabolites were involved in triggering ISR.

TABLE 1. Bacterial determinants of induced systemic resistance by fluorescent *Pseudomonas* spp. in different host plants

Determinant	*Pseudomonas* sp. strain	Host plant	Ref.
Cell envelope components			
Flagella	*P. putida* WCS358	*Arabidopsis*	23
Lipopolysaccharides	*P. fluorescens* WCS374	Radish	16
	P. fluorescens WCS417	*Arabidopsis*	46
		Carnation	44
		Radish	16
	P. putida WCS358	*Arabidopsis*	23
		Bean	23
		Tomato	23
Iron-regulated metabolites			
N-alkylated benzylamine derivative	*P. putida* BTP1	Bean	27
Pseudobactin siderophore	*P. fluorescens* CHA0	Tobacco	19
	P. fluorescens WCS374	Radish	14
	P. putida WCS358	*Arabidopsis*	23
	P. putida WCS358	Bean	23
	P. putida WCS358	Eucalyptus	34
	P. putida WCS358	Tomato	23
Salicylic acid	*P. aeruginosa* 7NSK2	Bean	5,6
	P. aeruginosa 7NSK2	Tobacco	4
	P. fluorescens P3 *pchBA*	Tobacco	20
Pyocyanin and pyochelin (and/or salicylic acid)	*P. aeruginosa* 7NSK2	Tomato	1
Unknown	*P. fluorescens* WCS374	Radish	14
	P. fluorescens WCS417	Radish	14
Antibiotics			
2,4-Diacetylphloroglucinol	*P. fluorescens* CHA0	*Arabidopsis*	12
		Tomato	38
	P. fluorescens Q2-87	*Arabidopsis*	51

Iron-regulated metabolites. Under conditions of low iron availability, most aerobic and facultative anaerobic microorganisms, including fluorescent *Pseudomonas* spp., produce low-molecular-weight Fe^{3+}-specific chelators, so-called siderophores. The siderophores sequester ferric ions in the environment and the ferrated siderophores are taken up by the microbial cells through specific recognition by membrane proteins (11). In several studies, mutants defective in siderophore production were less or no longer effective in suppressing disease when compared with their wild-type biocontrol strain. Competition for ferric iron between fluorescent pseudomonads and plant pathogens is considered the mode of action of these siderophores (18). However, a role for siderophores in ISR has been reported in several systems. A clear-cut role for siderophores in ISR was reported for *P. putida* WCS358 in suppression of bacterial wilt in *Eucalyptus urophylla*, caused by *Ralstonia solanacearum* (34). Infiltration of leaves with wild-type bacteria or the purified pseudobactin 358, the fluorescent siderophore of WCS358, resulted in significant reduction of bacterial wilt after infection with *R. solanacearum*, whereas the pseudobactin-minus mutant of the strain was not effective. For *P. fluorescens* WCS374, the situation was different in that wild-type bacteria, the pseudobactin mutant, and the purified pseudobactin were all effective in suppression of the disease, indicating redundancy of ISR-triggering traits of WCS374 in this system. Variability between strains has also been observed in other plant hosts. In *Arabidopsis*, the role of pseudobactin 358 production in ISR by *P. putida* WCS358 is not clear. In tomato, however, pseudobactin 358 triggers ISR while a pseudobactin mutant does not (23). On the other hand, pseudobactin 358 also triggers ISR in bean, but the pseudobactin mutant is as effective as the wild type, indicating redundancy of triggering traits (23). Pseudobactin-mediated ISR was also found to be effective against *Tobacco necrosis virus* in tobacco. A pseudobactin-minus mutant of *P. fluorescens* CHA0 was less effective in reducing numbers of viral lesions and lesion diameter than the parental strain (19). *P. putida* BTP1 induces ISR in bean against *Botrytis cinerea* only when grown under iron-limited conditions (26). The iron-regulated elicitor of ISR in BTP1 was recently characterized as an *N*-alkylated benzylamine derivative (27). Its action on the plant appears to involve stimulation of the lipoxygenase pathway after challenge (25). The observation of Leeman et al. (14) that lowering iron availability for *P. fluorescens* WCS374 and WCS417 increased the level of ISR-mediated disease suppression was explained as triggering of ISR by the siderophores of these strains. Whereas the purified pseudobactin of WCS374 did induce ISR in radish, pseudobactin of WCS417 did not. Moreover, mutants of the strains defective in pseudobactin production were as effective as the parental strains.

Interestingly, under conditions of iron limitation, the strains produce SA in vitro. This may explain the increased effectiveness of the strains upon iron limitation, because exogenous application of SA, even at the extremely low dose of 100 fg, to radish roots significantly reduced Fusarium wilt (14). This would suggest that ISR triggered by these strains depends on SA signaling in the plants. However, in *Arabidopsis*, ISR mediated by various *Pseudomonas* spp. strains that have the capacity to produce SA is independent of SA (35). Another PGPR that induces systemic resistance, *Serratia marcescens* 90-166, produces SA upon iron limitation; however, this metabolite is not involved in triggering ISR (31). Mutant analysis revealed that catechol siderophore biosynthesis genes in this strain are associated with ISR in cucumber (30).

Observations that bacterial SA production is not involved in ISR by PGPR could be explained by either suppression of SA production in the rhizosphere or the observation that SA is a precursor in the production of SA-containing siderophores, such

as pseudomonine in *P. fluorescens* WCS374 (22) and pyochelin in *P. aeruginosa* 7NSK2 (1). In the latter case, SA would not be excreted by the bacteria, but all SA would be utilized in siderophore biosynthesis. For ISR in bean, tomato, and tobacco by *P. aeruginosa* 7NSK2, the situation is even more complex. Although SA production appeared to play a role in ISR by 7NSK2, it is actually only for mutant KMPCH, which produces SA but is no longer able to incorporate it into pyochelin, that such a role could be established (1,4–6). Measuring SA levels on tomato roots colonized by either 7NSK2 or KMPCH suggested that mutant KMPCH does produce SA in the rhizosphere, but the wild-type strain does not (1). Triggering of ISR by the wild-type 7NSK2 is now postulated to depend on a combined action of pyochelin and the phenazine antibiotic pyocyanin. A mutant of 7NSK2 that lacks SA and pyochelin production no longer induces resistance, but neither does a mutant defective in pyocyanin biosynthesis trigger ISR in tomato against *B. cinerea*. A treatment with the combination of the two mutants did result in significant suppression of *B. cinerea* (1). The induction of resistance by mutant KMPCH, however, depends on SA. Additional support for induction of resistance by bacterially produced SA comes from a study in which the SA biosynthesis genes of *P. aeruginosa* PAO1 were expressed in the non-SA-producing *P. fluorescens* strain P3 and improved ISR in tobacco against *Tobacco necrosis virus* (20).

It is noteworthy that in many cases iron-regulated metabolites of pseudomonads are involved in ISR by these bacteria, whereas in earlier studies, effects of siderophores were thought to be confined to direct interactions between the pathogen and the biocontrol agents. Similarly, antibiotics that have a direct inhibitory effect on microbial pathogens may be involved in triggering ISR.

Antibiotics. The most impressive evidence for the involvement of antibiotics in natural disease suppression comes from the laboratories of D. M. Weller and L. S. Thomashow in Pullman (WA), where it was shown that 2,4-diacetylphloroglucinol (DAPG)-producing fluorescent pseudomonads play a key role in development of take-all decline (33,50). This effect of DAPG appears to be directly on the pathogen and not plant-mediated. In a study by Mazzola et al. (21), isolates of *Gaeumannomyces graminis* var. *tritici* that varied in their sensitivities to the antibiotics phenazine-1-carboxylic acid and DAPG were also differentially controlled by *Pseudomonas* spp. strains producing these antibiotics. If the mode of action of DAPG in wheat were plant-mediated, one would expect that isolates of the pathogen that are less sensitive to DAPG would be effectively controlled. In contrast, however, a role for DAPG in ISR was recently demonstrated in *Arabidopsis.* In this plant system, DAPG produced by *P. fluorescens* CHA0 is the key compound in ISR against *Peronospora parasitica* (12). Also in *Arabidopsis*, ISR against *P. syringae* pv. *tomato* by *P. fluorescens* Q2-87 depends on the production of DAPG (51). In both studies, application of the pure compound DAPG triggered ISR. The importance of DAPG production in ISR was further supported by observations that mutants that do not produce DAPG do not induce resistance, and ISR triggering is restored in complemented mutants (12,51). In tomato, *P. fluorescens* CHA0 induces resistance against the root-knot nematode *Meloidogyne javanica*. Also, in this case, DAPG appears to be the bacterial trigger of ISR, since a DAPG-minus mutant was not effective and effectiveness was restored by complementation of the mutant (38). Finally, in *P. aeruginosa* 7NSK2 the phenazine antibiotic pyocyanin is involved in ISR against *B. cinerea* in tomato (1), as described in detail in the section on iron-regulated metabolites.

Concluding remarks. In addition to testing of purified bacterial compounds, the use of well-defined mutants of *Pseudomonas* spp. has been instrumental in the identification of bacterial triggers of ISR. However, limitations of the mutant approach are evident from the observed redundancy in the traits involved. The plant innate immune system is able to recognize many determinants from different microorganisms and to converge these signals into an appropriate defense response (7). In this model, it is logical that different PGPR determinants are able to activate the ISR pathway. In several cases, knockout mutants in bacterial traits that are important for ISR still trigger ISR, since when one trigger is inactivated, others will activate the plant's response. Some studies have suggested that specific traits are not involved in ISR. However, redundancy may obscure the effectiveness of certain metabolites and traits. For instance, it was suggested that phenazine is not involved in ISR by *P. chlororaphis* strain 06 in tobacco, since a GacS mutant that lacks phenazine production was as effective in ISR as the parental strain (39). However, there might be several inducers of ISR in this strain and adding purified phenazine would have been very informative in this study.

Also for plant growth-promoting *Bacillus* spp., mechanisms of ISR have been studied (13). Bacterial production of the volatile 2,3-butanediol is the trigger of *Bacillus*-mediated ISR in *Arabidopsis*. The signaling pathway that is activated in this case depends on ethylene but is independent of SA and JA signaling (37). It would be interesting to investigate the capacity of plant growth-promoting *Pseudomonas* spp. to produce 2,3-butanediol and its possible involvement in ISR. Identification of bacterial traits that are involved in ISR relies on time-consuming bioassays in which suppression of disease symptoms and population dynamics of the pathogen are used as parameters. Development of indicator plants that contain a reporter gene that is expressed when ISR occurs would be instrumental in identifying additional bacterial triggers of ISR. The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis* revealed that root colonization by *P. fluorescens* WCS417r did not lead to transcriptional changes in the leaves, whereas in the roots there is a large set of genes that are differentially transcribed (48). One of the genes that was upregulated by WCS417r is the MYB72 transcription factor gene. This gene is of special interest since an *myb72* knockout mutant of *Arabidopsis* no longer expresses WCS417r-mediated ISR, indicating that it plays an important role in ISR signaling in the plant (47). Currently, *Arabidopsis* reporter lines are being constructed using *myb72* and other genes for which similar roles in ISR have been determined.

Improving the effectiveness of biological control by fluorescent *Pseudomonas* spp. may be established by using combinations of strains that have different mechanisms of disease suppression, such as competition for iron and ISR (3). Combining SA-dependent and SA-independent ISR is another possibility to increase effectiveness (45). Several *Pseudomonas* strains produce SA under conditions of low iron availability and potentially are able to induce the SA-dependent signal transduction pathway. However, ISR by these SA-producing strains does not appear to depend on SA, and it is speculated that in most cases the SA is channeled into SA-containing siderophores (1,22,35). Manipulating SA production in these bacteria by either uncoupling SA production from the biosynthesis of SA-containing siderophores (1) or by transfer of SA biosynthesis genes into non-SA-producers (20) seems effective. Production of SA by strains that already possess determinants that effectively trigger SA-independent ISR may create strains that induce both signal transduction pathways simultaneously.

Redundancy in ISR-eliciting determinants in PGPR on the one hand hampers elucidation of the involvement of these determinants, while on the other hand it may guarantee ISR robustness. If one determinant fails to elicit ISR, or is not produced under certain conditions, other traits can still be effective. In such cases it would be advantageous if the different traits are also differentially regulated. Increased knowledge on the variety of bacterial determinants of ISR and their regulation in the rhizosphere will not only increase our fundamental understanding of plant–microbe interactions in this highly dynamic environment, but also provide opportunities to exploit this mode of action of PGPR in crop protection strategies.

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